



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>7</sup> :</b> <b>C12N 15/00</b>	<b>A2</b>	<b>(11) International Publication Number:</b> <b>WO 00/18903</b> <b>(43) International Publication Date:</b> 6 April 2000 (06.04.00)
<b>(21) International Application Number:</b> PCT/US99/22468 <b>(22) International Filing Date:</b> 29 September 1999 (29.09.99)  <b>(30) Priority Data:</b> 60/102,140 29 September 1998 (29.09.98) US  <b>(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application</b> US 60/102,140 (CIP) Filed on 29 September 1998 (29.09.98)  <b>(71) Applicant (for all designated States except US):</b> THE JOHNS HOPKINS UNIVERSITY [US/US]; Suite 906, 111 Market Place, Baltimore, MD 21202 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> MARBAN, Eduardo [US/US]; The Johns Hopkins University, Suite 906, 111 Market Place, Baltimore, MD 21202 (US). JOHNS, David, C. [US/US]; The Johns Hopkins University, Suite 906, 111 Market Place, Baltimore, MD 21202 (US).		<b>(74) Agents:</b> KAGAN, Sarah, A. et al.; Banner & Witcoff, Ltd., 11th floor, 1001 G Street, N.W., Washington, DC 20001-4597 (US).  <b>(81) Designated States:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published:</b> <i>Without international search report and to be republished upon receipt of that report.</i>
<b>(54) Title:</b> INDUCIBLE GENETIC SUPPRESSION OF CELLULAR EXCITABILITY  <b>(57) Abstract</b>  Graded, reversible suppression of cellular excitability represents a logical goal of therapy for epilepsy and intractable pain, as well as for cardiac arrhythmias. To achieve such suppression, "electrical silencing" genes are transferred into cells with sensitive control of transgene expression. For example, an ecdysone-inducible promoter drives the expression inwardly rectifying potassium channels in polycistronic adenoviral vectors. While normal electrical activity is not affected, after the induction of gene expression excitability is suppressed.		

## INDUCIBLE GENETIC SUPPRESSION OF CELLULAR EXCITABILITY

This invention was made using funding supplied by the U.S. National Institutes of Health. Under the terms of NIH grant DA-00266, the U.S. government retains certain rights in the invention.

### **TECHNICAL FIELD OF THE INVENTION**

This invention is related to the field of cellular excitability. In particular it is related to the disorders in cellular excitability which lead to epilepsy, cardiac arrhythmias, and intractable pain.

### **BACKGROUND OF THE INVENTION**

Excessive cellular excitability underlies a variety of common, often lethal diseases, ranging from epilepsy to cardiac ventricular arrhythmias. Conventional therapeutic efforts to suppress excitability in such disorders center around pharmacological block of selected ion channels, mechanical disruption of an irritable focus, or devices such as pacemakers or defibrillators. Drug block of ion channels, while sometimes effective, is plagued by side effects attributable to the systemic administration of drugs needed only in small areas of the body. Mechanical disruption of epileptic or arrhythmic foci is traumatic, irreversible and often ineffective. Implantable devices designed to terminate hyperexcitation are expensive, and are activated only after the seizure has begun. Thus there is a need in the art for additional means of treating hyperexcitability which are more effective, less invasive, and less expensive.

Schematic representation of the basis for the ecdysone inducible adenovirus vectors; VgECR- modified ecdysone receptor, RXR- retinoid X receptor, ITR- inverted terminal repeat,  $\psi$ - packaging signal, ECD promoter- ecdysone-inducible promoter, IRES- internal ribosome entry site, pA- SV40 poly-adenylation signal, MCS- multiple cloning site (Fig. 1 A). Infection of A549 cells with variable multiplicities of infection (moi's) of both an adenovirus containing an ecdysone inducible promoter controlling expression of a cassette containing GFP, an internal ribosome entry site (IRES) and a multiple cloning site (AdEGI) and an adenovirus which expresses the modified ecdysone receptor and the retinoid X receptor (AdVgRXR) resulted in muristerone A dependent inducible expression of GFP (Fig. 1B, ■ vs • ). In the absence of AdVgRXR background expression was minimal (Fig. 1B, ▲) and such cells were entirely refractory to muristerone A (Fig. 1B, ○). Expression of GFP in A549 cells, infected with both AdVgRXR and AdEGI, can be induced with 3  $\mu$ M muristerone A for the indicated amounts of time (Fig. 1 C; columns 2-4), reduced to near background levels by the removal of muristerone A for 36 hours (Fig. 1 C; column 5) and re-induced to the original induction level with muristerone A for 24 hours (Fig. 1C; column 6). Membrane currents from A549 cells infected with AdVgRXR and AdEGI-Kir2.1 in the absence ( $I=-5$  pA/pF;  $n=2$ ) and presence ( $I=-80(12$  pA/pF;  $n=4$ ) of 3  $\mu$ M muristerone A (Fig. 1 D).

## Fig. 2: Infection of SCG neurons with inducible adenovirus constructs

Confocal images of SCG neurons infected with AdEGI-Kir2.1 and AdVgRXR in the absence (Fig. 2A) and presence (Fig. 2B) of muristerone A. Figs. 2C, 2D & 2E show a neuron expressing the fusion protein, EGFP-Kir2.1, following fixation and

## **DETAILED DESCRIPTION**

It is a discovery of the present inventors that inducible genetic suppression can be used as an alternative approach to the treatment and prevention of hyperexcitability. For example, overexpression of inwardly rectifying potassium channel genes driven by an inducible promoter inhibits evoked and spontaneous activity in cultured neurons. Blocking the expressed channels reverses the suppression of excitability. Thus, induction of exogenous hyperpolarizing genes effectively suppresses excitability. The inducible expression of such genes represents a new therapeutic principle for use in disorders of hyperexcitability, and provides a powerful research tool for probing the contributions of specific regions of the nervous system to the global functional response. Similarly, such inducible expression can be used *in vitro* or *in vivo* to screen for and identify new agents for enhancing or decreasing excitability or the suppression of excitability.

Broadly, the invention utilizes gene transfer of ion channels under the control of an inducible promoter to cells, tissues or organs to alter their electrical properties. Any gene delivery vector or vehicle can be used, so long as efficient gene transfer to cells occurs. Thus viral or non-viral vectors can be used. Similarly, naked DNA, DNA-coated particles, liposome encapsulated DNA, poly-lysine complexed DNA all can be used in the practice of the invention. Viral vectors which can be used include adenovirus, both partially and completely deleted, adeno-associated (AAV) vectors, herpes (HSV) vectors, both partially and completely deleted, and retro-viral vectors including lentiviral vectors. For a review of these vectors see Robbins et al., 1998, Trends Biotechnol 16, 35-40.

*Ther 3*, 900-912.) Suitable ion channels include, but are not limited to, potassium channels, including voltage gated, ligand gated and inwardly rectifying channels; sodium channels including voltage gated and ligand gated; calcium channels including voltage gated and ligand gated; chloride channels including voltage gated and ligand gated; non-specific cation channels; non-specific anion channels; and neurotransmitter-gated ion channels, *e.g.*, glutamate receptors.

Any tissue or cell which contains excitable membranes can be targeted by the method of the invention. Such tissues and cells include neuronal, muscle (cardiac, skeletal and smooth), and endocrine tissues and cells. In addition other tissues may be targeted in which altering the electrical properties creates a desired phenotype, such as cancerous tissue. The elicited effects include increasing or decreasing excitability as measured by electrical activity, secretory activity, contractile function, metabolic rate and growth status.

The method of the invention has benefit for treating a wide variety of diseases and pathological states. These include: heart failure, cardiac arrhythmias, endocrine disorders such as pituitary adenomata, epilepsy, intractable pain, skeletal muscle disorders including dystrophies, myotonia and ALS, heritable disorders of ion channels (long QT syndrome, heritable atrial fibrillation, cystic fibrosis, and others), hypertension, cancer, and Alzheimer's disease.

According to one preferred mode of the operation, inducible constructs according to the invention are delivered to intact hearts. By this method, one can increase or decrease refractoriness of the heart, increase or decrease the speed of conduction, increase or decrease focal automaticity, and/or alter the spatial pattern of excitation. Thus the invention can achieve modification of the sinus node and/or surrounding atrial

Brugada syndrome. In the atrium, one can use such treatments for treatment and prophylaxis of atrial fibrillation.

Neuronal excitability can be modified genetically in an inducible and reversible fashion. The ecdysone regulatory system, one of many possible inducible transcription systems which can be used, has little background activity when appropriate amounts of receptor virus are used and yet is inducible to high levels. Under the conditions described below, there was an inherently high level of variability of expression amongst infected cells. This was the result of co-infecting with less than saturating amounts of both the therapeutic virus and the receptor virus. Fig. 1B shows that increasing the number of the receptor viruses per cell, while holding the number of test viruses constant, increases the amount of expression. Similar experiments varying the number of test viruses delivered while holding the number of receptor virus constant showed that this also affected expression levels. The variable levels of GFP fluorescence visible in infected cells confirmed these predictions. For these reasons we did not perform dose response experiments in neurons, where the level of muristerone A was also varied. According to one embodiment of the invention, the hormone receptor complex genes can be included on the same vector as the inducible gene construct, optionally under the control of a tissue-specific promoter, to accomplish more uniform tissue-specific expression. The use of the internal ribosome entry site (IRES) was quite beneficial in allowing easy detection of positive cells without having to increase the amount of ion channel expression to levels which are not physiologically relevant. These properties enabled us to examine a wide range of electrophysiological effects.

On one end, low levels of expression of Kir2.1 sufficed to inhibit spontaneous activity (Fig. 3 G-I) despite a relatively modest 66% increase in the current needed to

## **EXAMPLES**

### **Materials and Methods used in the Examples**

#### **Plasmid Vectors-**

The coding sequence for the human Kir2.1 gene was amplified using the  
5 polymerase chain reaction to allow in frame fusion to EGFP in the vector pEGFP-C3  
(Clontech, Palo Alto, CA) creating pEGFP-Kir2.1. Kir2.1 was also cloned into the  
vector pGFP-IRES to create the vector pGFP-IRES-Kir2.1. The adenovirus shuttle  
vector pAdLox was modified to replace the CMV promoter with the ecdysone inducible  
promoter from pIND-1 (Invitrogen, San Diego, CA) making the vector pAdEcd. The  
10 expression cassettes from pGFP-IRES, pGFP-IRES-Kir2.1 and pEGFP-Kir2.1 were  
cloned into the multiple cloning site of pAdEcd making the vectors pAdEGI,  
pAdEGI-Kir2.1 and pAdEG-Kir2.1. The plasmid, pAdVgRXR, was made by cloning the  
expression cassettes from pVgRXR (Invitrogen, San Diego, CA) into pAdLox, encodes  
the ecdysone receptor.

#### **Cell Culture-**

15 A549, 293 and CRE8 cells (CCL-185, CRL 1573, American Type Culture  
Collection, Rockville, MD and respectively) were grown in DMEM (CellGro,  
Mediatech, Washington D.C.) supplemented with 10% fetal bovine serum (FBS), 2mM  
Glutamax, 1X Penicillin-Streptomycin, and 15 mM HEPES, pH 7.4 (Life Technologies,  
20 Gaithersburg, MD) at 37C° in a 5% CO<sub>2</sub>, humidified incubator.

## **Infections-**

Cells (A549 or primary SCG neurons) were infected by replacing their normal growth media with DMEM with 2% FBS and the appropriate amount of virus for 2-4. The amount of virus used for A549 cells was varied as described for each experiment, while the amount of virus used for SCG neurons was held constant at ~50 moi of the test virus and 5 moi of the receptor virus. Infection medium was then replaced with normal growth medium. Expression was induced by addition of muristerone A (1  $\mu$ M in Fig. 1B and 3  $\mu$ M in all other experiments; Invitrogen) for twenty-two (A549 cells) or 48 (SCG neurons) hours before assays were performed. The dose of muristerone A was selected to maximally activate the receptor based on previously reported dose-response curves (No et al), which agreed with our own preliminary dose-response curves (not shown).

**Immunohistochemistry-**Infected SCG neurons were fixed and stained as previously described.

## **Confocal Microscopy**

Images were taken on a laser scanning confocal microscope (PCM 2000, Nikon Inc., Melville, NY, EX 488, EM 505-530 ) with a 60X objective lens (NA 1.2).

## **Electrophysiology**

Experiments were performed at 21-23°C. Whole-cell currents were recorded with Axopatch 1-D amplifier (Axon Instruments, Foster City, CA) sampled at 10 kHz and filtered at 1-5 kHz. The pipette solution contained (in mM) 140 K-Glutamate, 1  $\text{MgCl}_2$ , 4 EGTA, 4  $\text{MgATP}$ , 10 HEPES (pH 7.2). The bath solution for neurons contained (in mM) 140 NaCl, 5.4 KCl, 2  $\text{CaCl}_2$ , 1  $\text{MgCl}_2$ , 10 glucose, 10 HEPES (pH7.4); for A549 cells 140 KCl was substituted for NaCl.



cytometer. The absolute level of expression was increased with increasing levels of the receptor. Removal and re-addition of muristerone A (Fig. 1C) could turn expression off and then on again. To test for inducibility with channel-containing constructs, A549 cells were co-infected with an adenovirus which expresses both GFP and Kir2.1 (AdEGI-Kir2.1; moi = 5) and AdVgRXR (moi = 0.5). When induced, 80-90% of the cells were GFP-positive in contrast to <1% of infected but non-induced cells (based on flow cytometry measurements; data not shown). Membrane current recordings from infected but non-induced cells showed no inwardly rectifying current, while induced cells expressing GFP exhibited robust inwardly-rectifying currents (Fig. 1 D). Overexpression of such channels under physiological conditions in neurons would be predicted to hyperpolarize cells and to suppress excitability.

## EXAMPLE 2

### **Infection of neuronal cells**

To confirm efficient infection and inducibility in excitable cells, we used neurons cultured from neonatal rat superior cervical ganglia (SCG). SCG neurons have been used extensively for studies of electrophysiology, biochemistry and development ; in addition, these neurons are known to be amenable to adenoviral gene transfer. SCG neurons infected with AdEGI-Kir2.1 and AdVgRXR for 48 hours and treated with vehicle for 22 hours exhibited negligible GFP fluorescence (Fig. 2A), in contrast with cells imaged 22 hours after the addition of muristerone A (Fig. 2B). A vector containing a GFP-Kir2.1 fusion protein (AdEG-Kir2.1) enabled us to track the distribution of the expressed channel complex. Comparison of the distribution of GFP (Fig. 2 D) to that of microtubule associated protein 2 (MAP2;

5 of these 14 neurons began firing spontaneously after superfusion with 50 (M Ba<sup>2+</sup>. The cell shown in Fig. 3G-I expressed only 100 pA of Ba<sup>2+</sup>-sensitive current at -120 mV, revealing that the amount of expression needed to achieve biologically relevant suppression was small. On the other hand, cells with as much as 1.1 nA of current also exhibited Ba<sup>2+</sup>-induced spontaneous activity, indicating that high-level expression did not irreversibly inhibit the ability of the neuron to respond to normal synaptic input.

Fig. 4 summarizes the changes in resting membrane potential (panel A) and stimulus threshold (B) effected by induction of Kir2.1 expression. In the absence of Ba<sup>2+</sup>, control cells were depolarized relative to Kir2.1-induced cells (-63 vs. -85 mV,  $p < 0.0001$ ). Some (but not all) of the control cells exhibited a small depolarization in response to Ba<sup>2+</sup>, due presumably to block of low-density native inward rectifiers. In contrast, every Kir2.1-induced cell depolarized upon exposure to Ba<sup>2+</sup> ( $p < 0.001$ ). Fig. 4B quantifies the change in excitability as the difference between the threshold amount of stimulus current needed to fire an action potential in the absence and presence of 50(M Ba<sup>2+</sup>. The stimulus threshold was Ba<sup>2+</sup>-insensitive in control cells. In general, Kir2.1-induced cells required dramatically greater stimuli to reach threshold in the absence of Ba<sup>2+</sup> ( $p < 0.0005$ ). To examine the effects of the varying densities of expressed Kir2.1, we divided the cells into two groups based on the amount of Ba<sup>2+</sup>-sensitive current they exhibited (600 pA was the midpoint; range = 100-1100 pA). The increase in stimulus threshold was distinctly greater in the cells which expressed a higher density of Kir2.1.

Gaithersburg, MD) at 37°C in a 5% CO<sub>2</sub> humidified incubator. Cells (A549 or primary SCG neurons) were infected by replacing their normal growth medium with DMEM with 2% FBS and the appropriate amount of virus for 2-4 hours. Infection medium was then replaced with normal growth medium. Expression was induced by addition of muristerone A (1-4 µM; Invitrogen) twenty-two (A549 cells) or 48 (SCG neurons) hours before assays were performed.

4. D.C. Johns et al., unpublished results.

5.  $I = -80 \pm 12$  pA/pF at -50mV;  $n=4$ . Experiments were performed at 21-23°C. Wholecell currents were recorded with Axopatch 1-D amplifier (Axon Instruments, Foster City, CA) sampled at 10 kHz and filtered at 1-5 kHz. The pipette solution contained (in mM) 140 K-Glutamate, 1 MgCl<sub>2</sub>, 4 EGTA, 4 MgATP, 10 HEPES (pH 7.2). The bath solution for neurons contained (in mM) 140 NaCl, 5.4 KCl, 2 CaCl<sub>2</sub>, 10 glucose, 10 HEPES (pH 7.4); for A549 cells 140 KCl was substituted for NaCl.

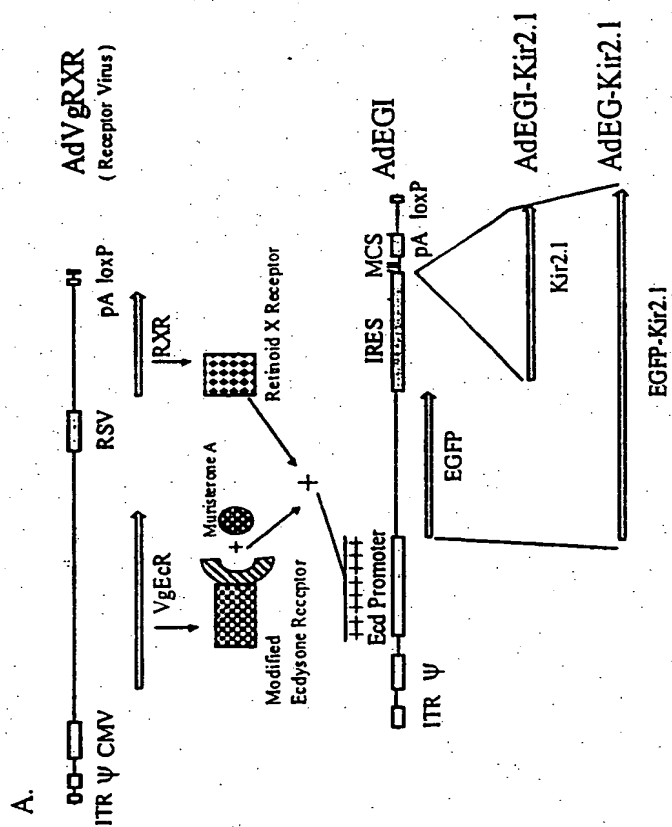
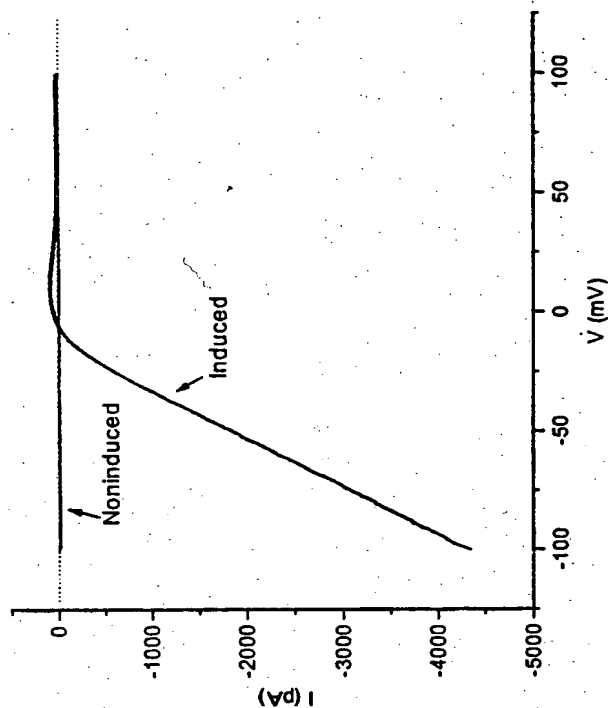
6. SCG neurons were prepared by enzymatic dissociation from ganglia taken from newborn rat pups [K.L. Marek, R.E. Mains, *J. Neurochem* 52, 1807-16 (1989)]. Neurons were plated onto glass coverslips coated with rat tail collagen [Bornstein, M.B., *Lab. Invest.* 7, 134-137 (1958)]. Neurons were grown in DMEM/F12 containing 10% FBS and treated with 10 µM cytosine arabinoside starting 24 hours after plating. Culture media contained β-nerve growth factor at 50 ng/ml (Boehringer Mannheim, Indianapolis, IN or kindly supplied by Dr. David Ginty, JHUMS, Baltimore, MD).

**We claim:**

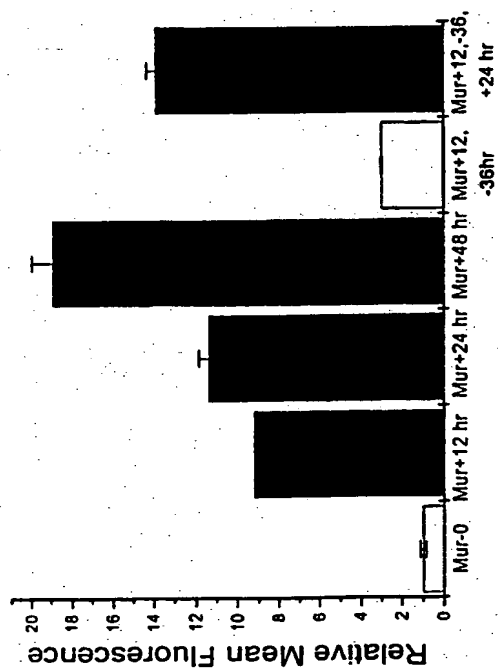
1. A method of altering cellular excitability, comprising the step of:  
delivering a genetic construct to a cell, said genetic construct comprising an  
inducible promoter and a sequence encoding an ion channel, wherein the inducible  
5 promoter regulates transcription of the sequence, wherein the inducible promoter is  
regulated by an externally controllable stimulus, whereby the excitability of the cell is  
regulated by the externally controllable stimulus.
2. The method of claim 1 wherein the ion channel is voltage gated.
3. The method of claim 1 wherein the ion channel is ligand gated.
- 10 4. The method of claim 1 wherein the ion channel is inwardly rectifying.
5. The method of claim 1 wherein the ion channel is a potassium channel.
6. The method of claim 1 wherein the ion channel is neurotransmitter gated.
7. The method of claim 1 wherein the cell is a neuron.
8. The method of claim 1 wherein the cell is a muscle cell.
- 15 9. The method of claim 8 wherein the cell is a cardiac muscle cell.
10. The method of claim 8 wherein the cell is a skeletal muscle cell.
11. The method of claim 8 wherein the cell is a smooth muscle cell.
12. The method of claim 1 wherein the cell is an endocrine cell.
13. The method of claim 1 wherein the cell is a cancer cell.
- 20 14. The method of claim 1 wherein the inducible promoter is regulated by a  
hormone.
15. The method of claim 1 wherein the inducible promoter is regulated by a  
hormone analogue.

28. The method of claim 25 wherein the genetic construct is part of a herpes virus vector.
29. The method of claim 25 wherein the genetic construct is part of a retrovirus vector.
- 5 30. The method of claim 29 wherein the genetic construct is part of a lentivirus vector.
31. The method of claim 1 wherein the delivery is to a muscle.
32. The method of claim 1 wherein the genetic construct is delivered as free DNA.
- 10 33. The method of claim 1 wherein the genetic construct is delivered in a liposome.
34. The method of claim 1 wherein the genetic construct is delivered by particle bombardment.
35. A method of altering cellular excitability, comprising the step of:
- 15 delivering a genetic construct to a neuronal cell, said genetic construct comprising an ecdysone-inducible promoter and a sequence encoding a potassium ion channel, wherein the inducible promoter regulates transcription of the sequence, wherein the inducible promoter is regulated by muristerone A, whereby the excitability of the cell is regulated by muristerone A.
- 20 36. A genetic construct for delivery to a neuronal cell, said genetic construct comprising:
- a sequence encoding a potassium ion channel which is inwardly rectifying; and
- an ecdysone-inducible promoter;

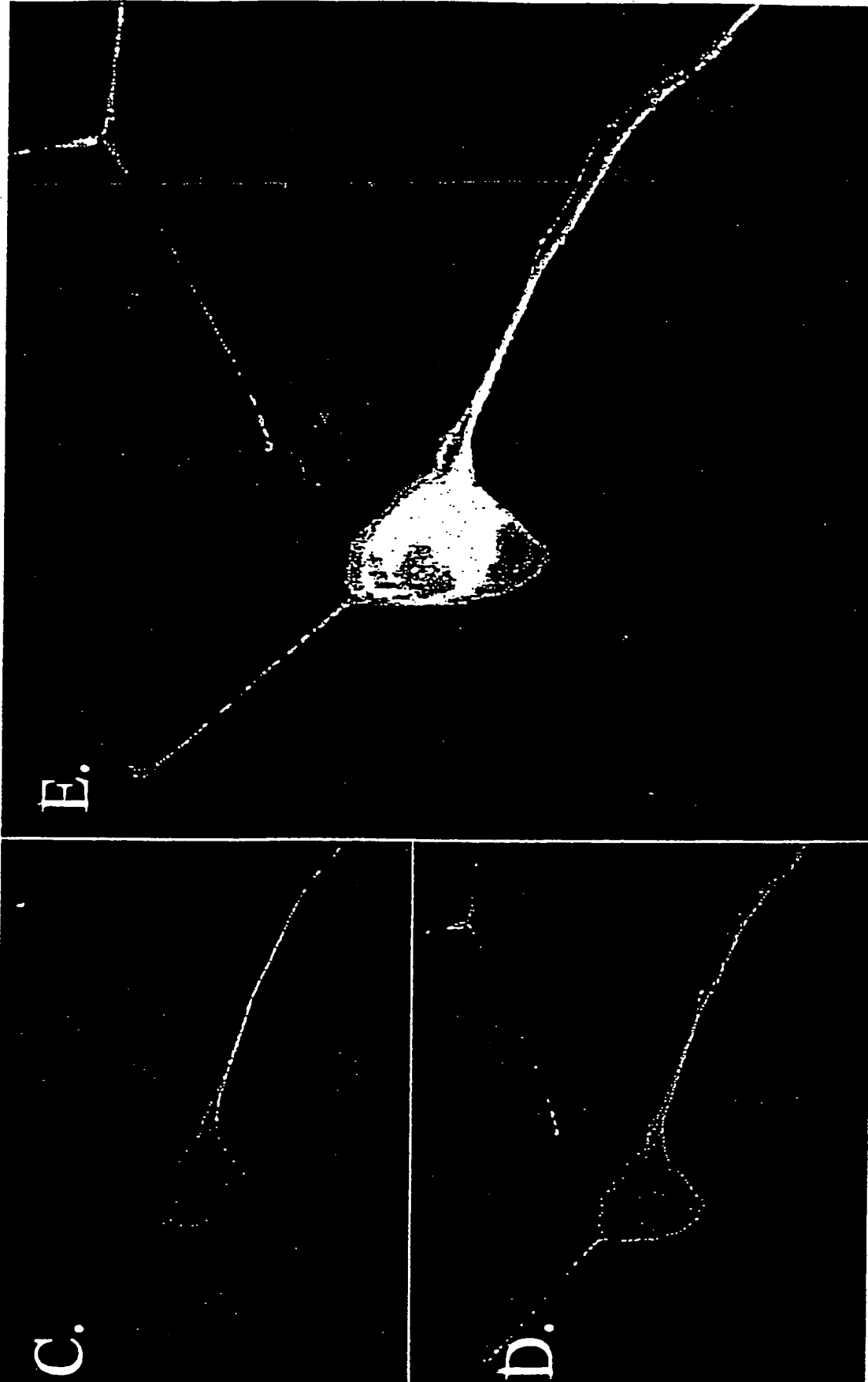
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### Figure 1 Time Course of Induction and Re-induction



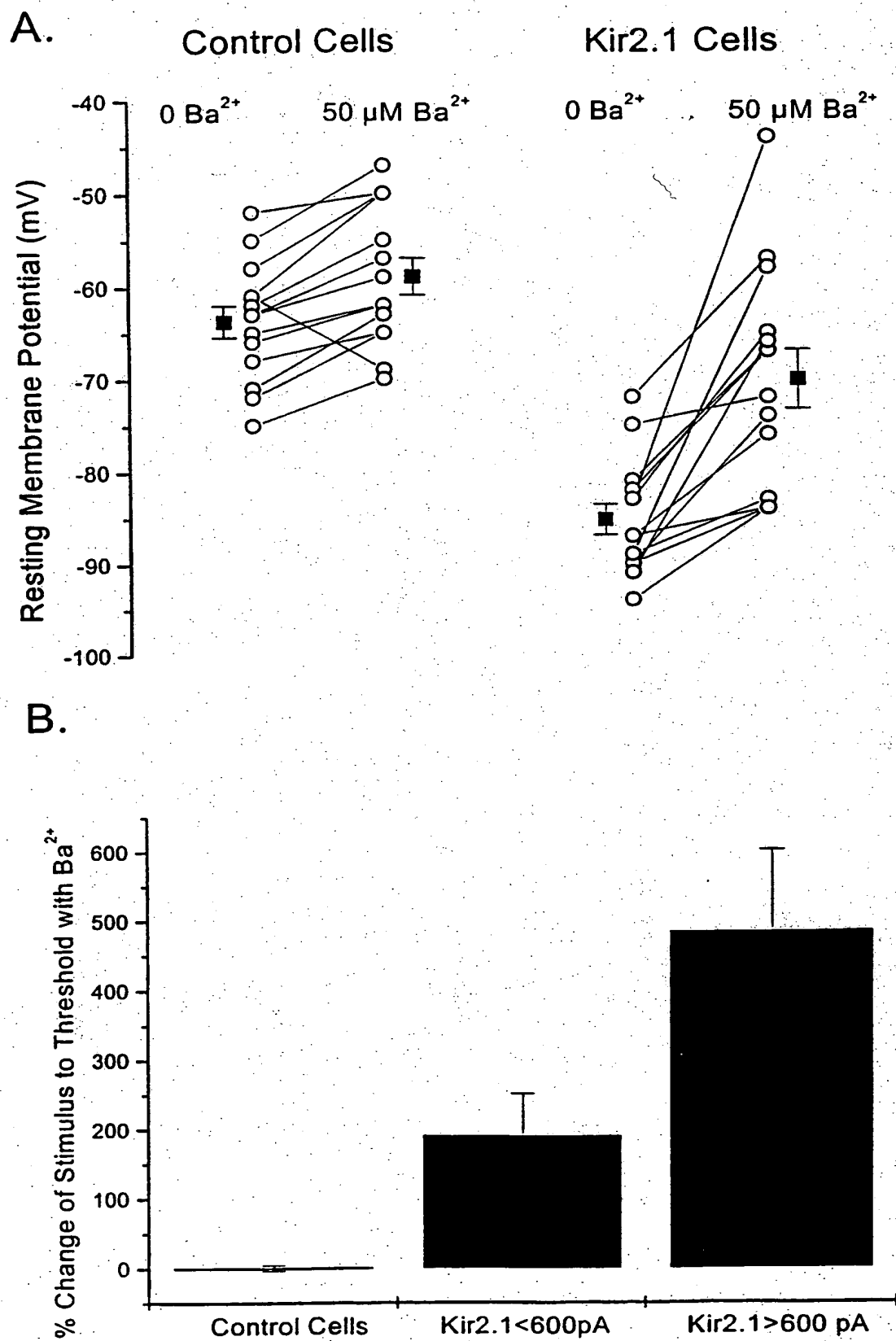


Figure 4





## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US99/22468 <b>(22) International Filing Date:</b> 29 September 1999 (29.09.99)  <b>(30) Priority Data:</b> 60/102,140 29 September 1998 (29.09.98) US  <b>(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application</b> US 60/102,140 (CIP) Filed on 29 September 1998 (29.09.98)  <b>(71) Applicant (for all designated States except US):</b> THE JOHNS HOPKINS UNIVERSITY [US/US]; Suite 906, 111 Market Place, Baltimore, MD 21202 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> MARBAN, Eduardo [US/US]; The Johns Hopkins University, Suite 906, 111 Market Place, Baltimore, MD 21202 (US). JOHNS, David, C. [US/US]; The Johns Hopkins University, Suite 906, 111 Market Place, Baltimore, MD 21202 (US).		<b>(74) Agents:</b> KAGAN, Sarah, A. et al.; Banner & Witcoff, Ltd., 11th floor, 1001 G Street, N.W., Washington, DC 20001-4597 (US).  <b>(81) Designated States:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>  <b>(88) Date of publication of the international search report:</b> 6 July 2000 (06.07.00)

**(54) Title:** INDUCIBLE GENETIC SUPPRESSION OF CELLULAR EXCITABILITY**(57) Abstract**

Graded, reversible suppression of cellular excitability represents a logical goal of therapy for epilepsy and intractable pain, as well as for cardiac arrhythmias. To achieve such suppression, "electrical silencing" genes are transferred into cells with sensitive control of transgene expression. For example, an ecdysone-inducible promoter drives the expression inwardly rectifying potassium channels in polycistronic adenoviral vectors. While normal electrical activity is not affected, after the induction of gene expression excitability is suppressed.

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PCT/US 99/22468

According to International Patent Classification (IPC) or to both national classification and IPC

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

### C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>HAN JIN-HEE ET AL: "Expression of noninactivating K<sup>+</sup> channels (aKv5.1) into Aplysia neuron using heat-shock inducible promoters and the study of its effect on neuronal activities."</p> <p>FOURTH MEETING OF THE ASIAN-PACIFIC SOCIETY FOR NEUROCHEMISTRY AND THE ANNUAL MEETING OF THE KOREAN SOCIETY FOR NEUROSCIENCE; SEOUL, KOREA; JUNE 24-26, 1998, vol. 70, no. SUPPL. 2, 1998, page S20-XP000885155</p> <p>Journal of Neurochemistry 1998</p> <p>ISSN: 0022-3042</p> <p>abstract</p>	1-34
Y	<p>—</p> <p>—/—</p>	35-37

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

• **Special categories of cited documents :**

- \* "document defining the general state of the art which is not considered to be of particular relevance"
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Date of the actual completion of the international search

**26 April 2000**

Date of mailing of the international search report

16/05/2000

Name and mailing address of the ISA

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Fax: (+31-70) 340-3016

Authorized officer

Sitch, W

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/22468

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>GINGRICH JEFFREY R ET AL: "Inducible gene expression in the nervous system of transgenic mice."  ANNUAL REVIEW OF NEUROSCIENCE ANNUAL REVIEW OF NEUROSCIENCE. 1998 ANNUAL REVIEWS INC. P.O. BOX 10139, 4139 EL CAMINO WAY, PALO ALTO, CALIFORNIA 94306, USA,  vol. 21, 1998, pages 377-405, XP000891476  ISBN: 0-8243-2421-8  cited in the application</p>	
P,X	<p>JOHNS DAVID C ET AL: "Inducible genetic suppression of neuronal excitability."  JOURNAL OF NEUROSCIENCE MARCH 1, 1999, vol. 19, no. 5, 1 March 1999 (1999-03-01), pages 1691-1697, XP000891377.  ISSN: 0270-6474  cited in the application  the whole document</p>	1-37

CORRECTED VERSION

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(54) Title: INDUCIBLE GENETIC SUPPRESSION OF CELLULAR EXCITABILITY

(57) Abstract: Graded, reversible suppression of cellular excitability represents a logical goal of therapy for epilepsy and intractable pain, as well as for cardiac arrhythmias. To achieve such suppression, "electrical silencing" genes are transferred into cells with sensitive control of transgene expression. For example, an ecdysone-inducible promoter drives the expression inwardly rectifying potassium channels in polycistronic adenoviral vectors. While normal electrical activity is not affected, after the induction of gene expression excitability is suppressed.

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## **SUMMARY OF THE INVENTION**

It is an object of the present invention to provide methods for altering the excitability of cells.

It is another object of the present invention to provide methods for altering the excitability of cells, such as those involved in epilepsy, cardiac arrhythmia, and intractable pain.

These and other objects of the invention are achieved by providing a method of altering cellular excitability. A genetic construct is delivered to a cell. The genetic construct comprises an inducible promoter and a sequence encoding an ion channel. The inducible promoter regulates transcription of the sequence. The inducible promoter is regulated by an externally controllable stimulus. Thus, the excitability of the cell is regulated by the externally controllable stimulus.

According to another embodiment of the invention, a method is provided for altering cellular excitability. A genetic construct is delivered to a neuronal cell. The genetic construct comprises an ecdysone-inducible promoter and a sequence encoding a potassium ion channel. The inducible promoter regulates transcription of the sequence. The inducible promoter is regulated by muristerone A and similar ligands such as ponasterone. Thus, the excitability of the neuronal cell is regulated by muristerone A.

These and other embodiments provide the art with new methods and tools for counteracting pathological hyperexcitability of cells.

## **BRIEF DESCRIPTION OF THE DRAWINGS**

**Fig. 1: Ecdysone inducible adenovirus constructs allow tight control of gene expression**

staining with a polyclonal antibody to GFP and a monoclonal antibody against the microtubule associated protein 2 (MAP2). A FITC labeled goat anti-rabbit secondary antibody was used to detect the presence of GFP (Fig. 2D) and a Cy3 conjugated secondary antibody was used to detect the bound anti-MAP2 (Fig. 2C). MAP2 is normally distributed in the dendrites and cell bodies.

### **Fig. 3: Exogenous expression of Kir2.1 suppresses excitability**

The effects of a supra-threshold stimulus on a control cell do not change with the addition of barium (Fig. 3 A, Fig. 3 B). The membrane current recorded from the same cell under these conditions using a ramp protocol from  $-138$  mV to  $+22$  mV over 500 ms (Fig. 3 C). The addition of barium had little or no detectable effect on the outwardly rectifying I-V curves in control cells. The response to a sub-threshold stimulus on a Kir2.1 infected cell (Fig. 3 D) is markedly changed by the addition of barium (Fig. 3 E), in both panels, the arrow indicates the cessation of the applied stimulus. The I-V curve for this cell is much different upon addition of barium (Fig. 3 F). Membrane current recordings from a Kir2.1 infected cell shown in normal saline (Fig. 3 G), upon superfusion with saline containing barium (Fig. 3H) and upon washout (Fig. 3I).

### **Fig. 4: Cumulative effects of exogenous expression of Kir2.1**

Cells expressing Kir2.1 are hyperpolarized with respect to control cells. This effect is reversed upon blocking the Kir2.1 channels (Fig. 4A). Increasing amounts of Kir2.1 current increase the size of the stimulus needed to fire an action potential (Fig. 4B).

Any mode of delivery can be used, so long as effective gene transfer is accomplished to the desired target cells. Thus delivery can be accomplished to cells *in vitro*, or alternatively delivery may be intramuscular, subcutaneous, intravascular, intrathecal, intravenous, etc. Catheters may be used to deliver the constructs of the invention to the desired location. These include magnetically-localizable catheters and injection catheters.

Inducible promoters which may be used in performing the methods of the present invention include those regulated by hormones and hormone analogs such as progesterone, ecdysone and glucocorticoids as well as promoters which are regulated by tetracycline, heat shock, heavy metal ions, interferon, and lactose operon activating compounds. For review of these systems see Gingrich and Roder, 1998, *Annu Rev Neurosci* 21, 377-405. When using non-mammalian induction systems, both an inducible promoter and a gene encoding the receptor protein for the inducing ligand are employed. The receptor protein typically binds to the inducing ligand and then directly or indirectly activates transcription at the inducible promoter.

Ion channels which can be used in the practice of the present invention include all proteins, natural and synthetic, which allow ionic currents to flow across a lipid bilayer membrane. These include proteins which are found in the plasma membrane as well as in intracellular and mitochondrial membranes. The proteins may be in their native form or may have been altered by molecular genetics to make them fit a specific need (*i.e.*, altering the activation or inactivation properties or altering the protein so that it acts as a dominant-negative suppressor as demonstrated in Johns et al, 1999, *J Neurosci* 19, 1691-7; Johns et al., 1995, *J Clin Invest* 96,1152-8; Johns et al., 1997, *J Biol Chem* 272,31598-31603; Nuss et al., 1999, *J Clin Invest* 103,889-96; Nuss et al., 1996, *Gene*

tissue. This can be used to eliminate inappropriate sinus tachycardia, *e.g.*, by introducing potassium channel genes to decrease nodal excitability. Introduction of inducible ion channel genes into focal areas of the heart can be used to create regions of automaticity. This is useful for indications such as sick sinus syndrome, Stokes-Adams attacks, syncope, chronic fatigue syndrome, and cardiomyopathies (hypertrophic and dilated). Introduction of HCN genes and/or calcium channel subunits can increase local automaticity and/or induce pacemaker activity where it is not normally present.

Introduction of inducible ion channel genes can also be used to slow and/or block conduction. For example, this can be achieved by overexpression of K channels, decrease of sodium or calcium channel activity (*e.g.*, by decoy beta subunits), or manipulation of cell-cell communication (*e.g.*, by dominant-negative or nonfunctional connexins). In the AV node, one can achieve modification of atrioventricular conduction velocity and/or pattern, *e.g.*, to control ventricular rate in atrial fibrillation, or to modify conduction in AV nodal reentrant tachycardia. In the tricuspid annulus, one can induce bidirectional block and prevent atrial flutter. In the atrium, one can induce lines of conduction block or slowing to terminate or to prevent atrial fibrillation (*e.g.*, a genetic "maze" procedure). In the ventricle, one can induce bundle-branch block as a means of achieving discoordinate activation, *e.g.*, in hypertrophic cardiomyopathy.

In order to achieve modification of repolarization, widespread rather than focal delivery is preferred. This is achievable by overexpression or functional knockout of K channels (*e.g.*, by dominant-negative constructs), or by alteration of sodium or calcium channel activity (*e.g.*, by decoy beta subunits). In the ventricle one can use such treatments for treatment of long QT syndrome (acquired or hereditary), heart failure, or



reach threshold. At the other extreme, two cells, which were not included in the analysis, exhibited  $Ba^{2+}$ -sensitive currents  $> 1.5$  nA. In these two cells, action potentials could not be elicited when a maximal current stimulus (2 nA) was applied for up to 50 ms; nevertheless, in the presence of  $Ba^{2+}$  both of these neurons fired action potentials with modest stimuli. Such stimulus currents far exceed the probable summed inputs for any single neuron and thus represent a complete silencing of electrical activity.

The higher infection efficiency of the glial cell population (100%) vs the neuronal population (52%) has been previously observed by us as well as others. This represents another potential limitation of this system, in that there may indeed be profound effects of hyperpolarizing the supporting cell population. To avoid this complication *in-vivo*, several strategies are employed. These include the use of neuronal-specific promoters, of vectors modified to increase neuronal tropism, or of vectors which are naturally more neurotropic.

The potential applications for this technology are widespread. The role of ion channels in nervous system development has previously been explored by the use of blocking reagents. The current methods enable genetic manipulation of excitability at different points in development and potentially in specific regions of the CNS. From a clinical standpoint, this targeted and controllable delivery of ion channels form the basis for new treatments of diseases of excitability such as epilepsy. In addition, targeted delivery of these (or similar) vectors results in controllable suppression of excitability with the potential for genetic analgesia in the treatment of intractable pain. The ability to turn gene expression on and off at will enables the titration of responses while preserving remarkable safety; should therapy prove detrimental or ineffective in a particular patient, the inducing agent can simply be withdrawn.

### **Viral Vectors-**

Recombinant adenovirus vectors were generated by Cre-lox recombination. A 25 cm<sup>2</sup> flask (T25, Sarstedt, Newton, NC) of CRE8 cells was co-transfected with 2.1 (g of purified (5 viral DNA and 2.1 (g of purified shuttle vector DNA using Lipofectamine Plus (Life Technologies). Cells were incubated 5 to 9 days until cytopathic effects (CPE) were observed. Cells and media were transferred to a 50 ml polypropylene tube (Sarstedt) and freeze-thawed in a dry ice-ethanol bath and shaking 37 C° water bath. Cells and debris were removed by centrifugation in a clinical centrifuge and two ml of the supernatant was added to a 90% confluent T25 and returned to the incubator until CPEs were observed. This procedure was repeated three to four times at which time the virus was analyzed for purity by plaque assays. Viruses were then expanded and large-scale virus purifications were performed as previously described. Virus titers were determined by plaque assays and particle numbers were determined by absorbance at 260 nm on a DU640 spectrophotometer (Beckman Instruments, Fullerton, CA). As has previously been reported, the particle number exceeded the infective particle number by between 40 and 100 fold. Multiplicities of infections were calculated based on the number of plaque forming units per ml.

### **Neuronal Cultures-**

SCG neurons were prepared by enzymatic dissociation from ganglia taken from newborn rat pups. Neurons were plated onto glass coverslips coated with rat tail collagen. Neurons were grown in DMEM/F12 containing 10% FBS and treated with 10 (M cytosine arabinoside starting 24 hours after plating. Culture media contained (-nerve growth factor at 100 ng/ml (Boehringer Mannheim, Indianapolis, IN or kindly supplied by Dr. David Ginty, JHUMS, Baltimore, MD).

## Statistics

Pooled data are shown as means ( the standard error of the mean (s.e.). Data was analyzed using two-factor ANOVA. Between group comparisons were made post hoc with the Fisher's least-significant-difference test.

## Flow Cytometry

Single color flow cytometry was used to determine the mean fluorescence intensity of infected A549 cells. Cells were induced one day after infection, twenty-two hours later, cells were trypsinized, washed and re-suspended in 0.5 ml of PBS. Cells were analyzed on a FacStar (Becton Dickinson) using forward and side scatter gates to exclude dead cells and cell fragments and collecting  $1 \times 10^4$  events per sample.

## EXAMPLE 1

### Inducible viral constructs

To achieve sensitive control of gene expression after somatic gene transfer, we created adenoviruses that enabled ecdysone-inducible expression of ion channels and reporter genes in mono- or polycistronic vectors (Fig. 1A). The poly-cistronic vector, as shown, results in an approximate 3:1 ratio of GFP protein to ion channel protein, allowing sensitive detection of cells without undue overexpression of the ion channel. Infection of A549 cells with a stoichiometric ratio of 10 ecdysone-driven reporter virus (AdEGI) per ecdysone/retinoid X receptor viruses (AdVgRXR) produced low basal levels of expression of green fluorescent protein (GFP), but >15-fold enhancement after addition of the ecdysone analog muristerone A (Fig. 1B). Excess levels of receptor virus lead to higher levels of basal (noninduced) expression (Fig. 1B) as well as a slight decrease in the fold induction achievable. This decrease in the level of induction may be in part due to a higher percentage of induced cells above the saturation level of the flow

panel 2 C) showed substantial overlap (panel 2 E); indeed, Kir2.1 protein was distributed throughout the surface and internal membranes of the neurons. In these experiments, 52% of the MAP-2 positive cells were also GFP positive (N=100); in addition, all of the glial cells that could be identified by visible light were GFP positive.

### EXAMPLE 3

#### **Suppression of neuronal excitability**

In the process of characterizing the phenotypic consequences of Kir2.1 gene transfer and induction, we first compared action potentials and membrane currents in AdEGI-infected and uninfected SCG neurons, and found no differences; data from these two groups were thus combined as "control cells" (data not shown). Barium ( $Ba^{2+}$ ) selectively blocks inwardly rectifying potassium channels, which are relatively sparse in control SCG neurons. Stimulated action potentials in a control cell were identical in the absence and presence of  $Ba^{2+}$  (Fig. 3A,B), as were membrane currents (Fig. 3C). In contrast, neurons expressing Kir2.1 exhibited diminished excitability. Inward current injection produced only a sub-threshold depolarization in an induced cell infected with AdEGI-Kir2.1 (Fig. 3D); after the addition of 50 (M  $Ba^{2+}$ , the resting potential depolarized and a current stimulus of the same intensity evoked a full action potential (Fig. 3E). The current-voltage relations in this cell reveal marked inward rectification at baseline; the addition of  $Ba^{2+}$  made the membrane currents indistinguishable from those in control cells (Fig. 3F). In addition to re-shaping evoked activity, induced Kir2.1 gene expression also suppressed spontaneous electrical activity (Fig. 3 G-I). Six of 14 control cells exhibited spontaneous activity in normal solution, but none of 14 induced Kir2.1-infected cells did so. Nevertheless,

## References and Notes

1. W. Loscher, *Eur J. Pharmacol* 342, 1-13, 1998; P. Jallon, *Epilepsia* 38, S37-42, 1997; E. Marban in *Molecular basis of heart disease*. K. Chien et al., eds., WB Saunders, NY, 1998
2. Adenovirus constructs were created using a CRE-Lox recombination system [S. Hardy et al., *J. Virol.* 71 1842-1849 (1997)]. The ecdysone-inducible promoter [D. No, T.-P. Yao, R. M. Evans, *Proc. Natl. Acad. Sci. USA* 93, 3346-3351 (1996)] was cloned from pInd (Invitrogen, San Diego, CA) into pAdLox to create pAdEcd. The coding sequence for the human Kir2.1 gene was amplified using the polymerase chain reaction to allow in frame fusion to EGFP in the vector pEGFP-C3 (Clontech, Palo Alto, CA) creating pEGFP-Kir2.1. Kir2.1 was also cloned into the vector pGFP-IRES [D.C. Johns et al, *J. Biol. Chem.* 272, 31598-31603 (1997)] to create the vector pGFP-IRES-Kir2.1. The coding sequences from these two vectors and pGFP-IRES were cloned into pAdEcd and used to generate the adenoviruses AdEG-Kir2.1, AdEGI-Kir2.1, and AdEGI respectively. The dual expression cassette from pVgRXR (Invitrogen, Can Diego, CA) was also cloned into pAdLox and subsequently used to generate the virus AdVgRXR.
3. A549, 293 and CRE8 cells (CCL-185, CRL 1573, American Type Culture Collection, Rockville, MD and kindly supplied by S. Hardy, respectively) were grown in DMEM (CellGro, Mediatech, Washington D.C.) supplemented with 10% fetal bovine serum (FBS), 2mM Glutamine, 1X Penicillin-Streptomycin, and 15 mM HEPES, pH 7.4 (Life Technologies,

7. V. Argiro, M.I. Johnson, *J. Neurosci* **2**, 503-12 (1982); T.K. Garyantes, W.G. Regehr, *J. Neurosci* **12**, 96-103 (1992); S. McFarlane, E. Cooper, *J. Neurophysiol* **67**, 1291-300 (1992); J.M. Nerbonne, A.M. Gurney, *J. Neurosci* **9**, 3272-86 (1989); S.T. Lockhart, G.G. Turrigiano, S.J. Birren, *J. Neurosci* **17**, 9573-82 (1997); H.S. Wang, D. McKinnon, *J. Physiol (Lond)* **485**, 319-35 (1995).
8. L. Paquet, B. Massie, R.E. Mains, *J. Neurosci* **16**, 964-73 (1996).
9. Seven non-infected cells were compared to seven cells infected with AdEGI and AdVgRXR (data not shown).
10. H.S. Wang, D. McKinnon, *J. Physiol (Lond)* **492**, 467-78 (1996).
11. This effect was not statistically significant ( $p=0.14$ ).
12. The effects of different levels of expression of the Kir2.1 protein were probed by comparing cells infected either with the polycistronic construct (AdEGI-Kir2.1) or the fusion construct (AdEG-Kir2.1), which produced different levels of GFP fluorescence and current density.
13. P. Rakic, H. Komuro, *J. Neurobiol* **26**, 299-315 (1995); S.B. Kater, L.R. Mills, *J. Neurosci* **11**, 891-9 (1991).
14. C.G. Wasterlain, Y. Shirasaka, A.M. Mazarati, I. Spigelman, *Epilepsy Res* **26**, 255-65 (1996).
15. A. Dray & H. Rang, *Trends in Neurosci.* **21** 315-317 (1998).

16. The method of claim 1 wherein the inducible promoter is regulated by an antibiotic.

17. The method of claim 16 wherein the inducible promoter is regulated by tetracycline.

5 18. The method of claim 1 wherein the inducible promoter is regulated by heat shock.

19. The method of claim 1 wherein the inducible promoter is regulated by a heavy metal ion.

10 20. The method of claim 1 wherein the inducible promoter is regulated by a cytokine.

21. The method of claim 1 wherein the inducible promoter is regulated by an interferon.

22. The method of claim 1 wherein the inducible promoter is regulated by a lactose operon inducer.

15 23. The method of claim 22 wherein the inducible promoter is regulated by IPTG.

24. The method of claim 22 wherein the inducible promoter is regulated by lactose.

20 25. The method of claim 1 wherein the genetic construct is part of a viral vector.

26. The method of claim 25 wherein the genetic construct is part of an adenovirus vector.

27. The method of claim 25 wherein the genetic construct is part of an adeno-associated vector.

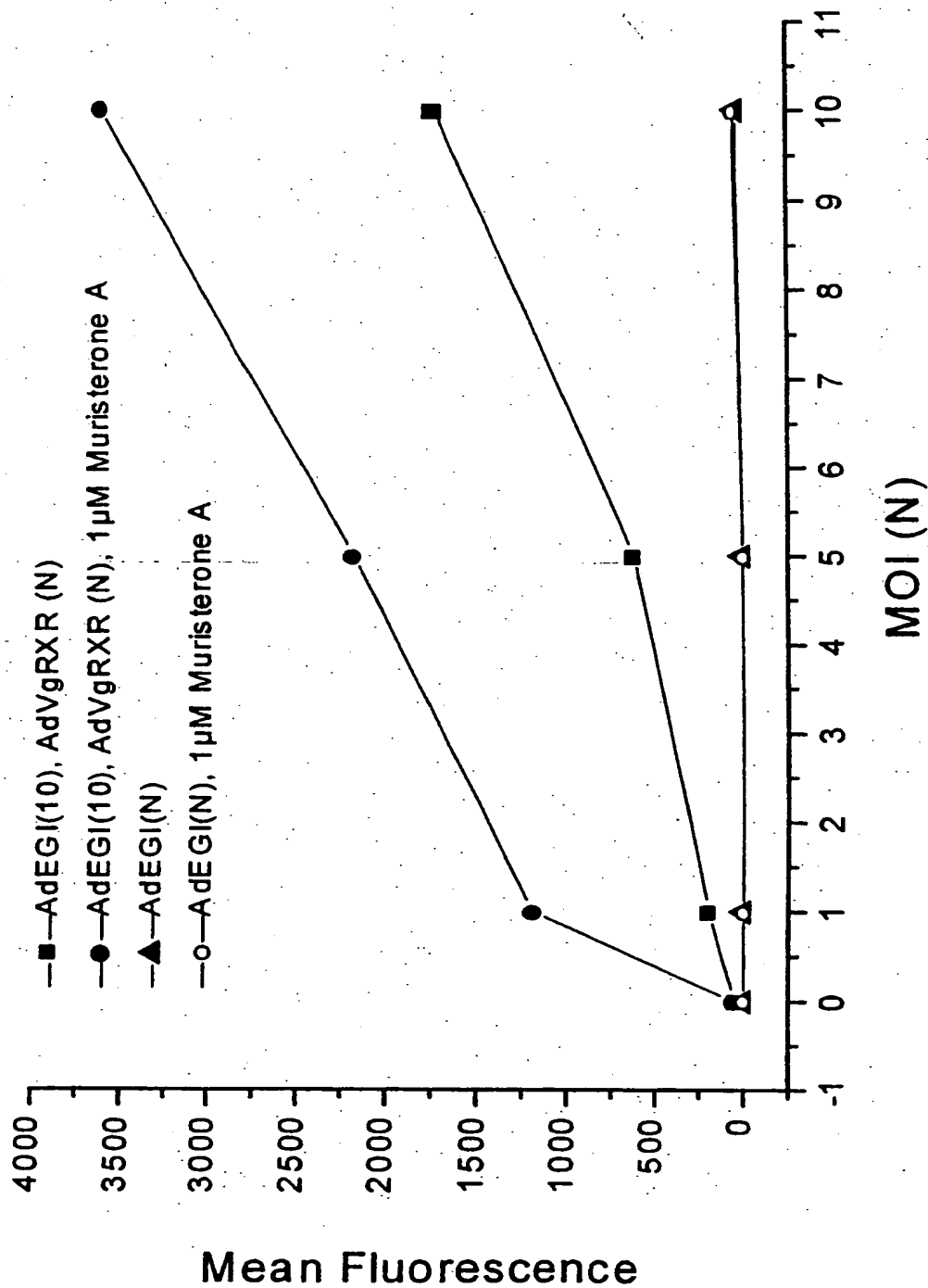
wherein the ecdysone-inducible promoter regulates transcription of the sequence, and  
wherein the ecdysone-inducible promoter is regulatable by muristerone A.

37. The genetic construct of claim 36 which is contained in an adenoviral vector.



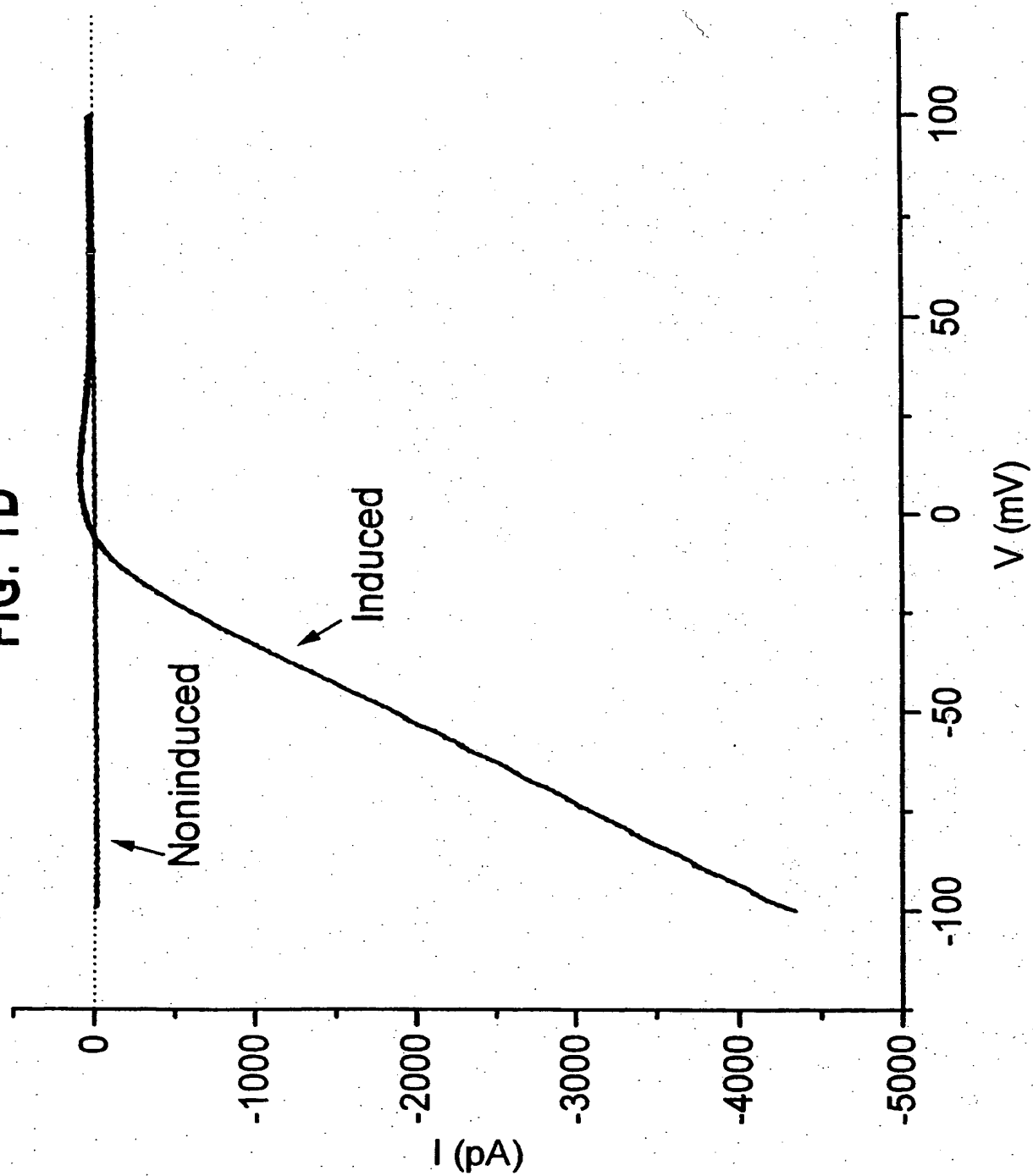
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FIG. 1B



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FIG. 1D



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FIG. 3C

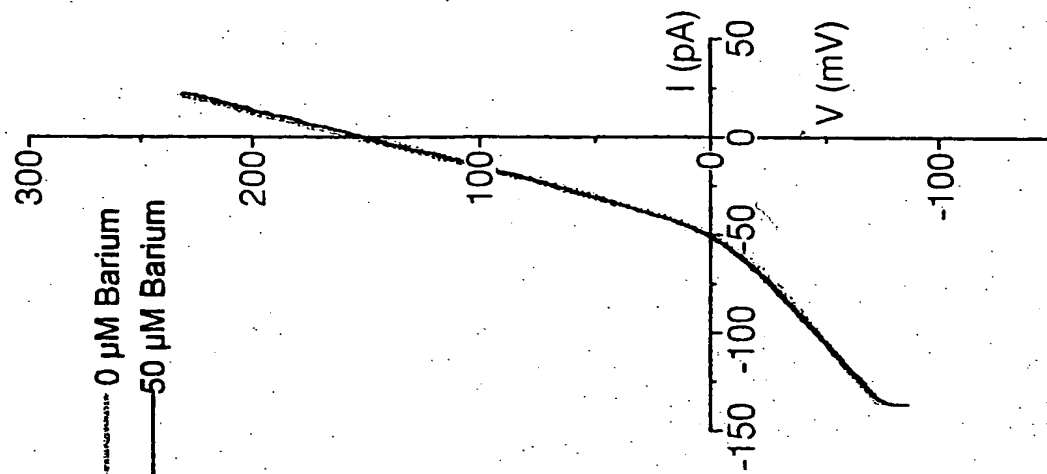


FIG. 3B

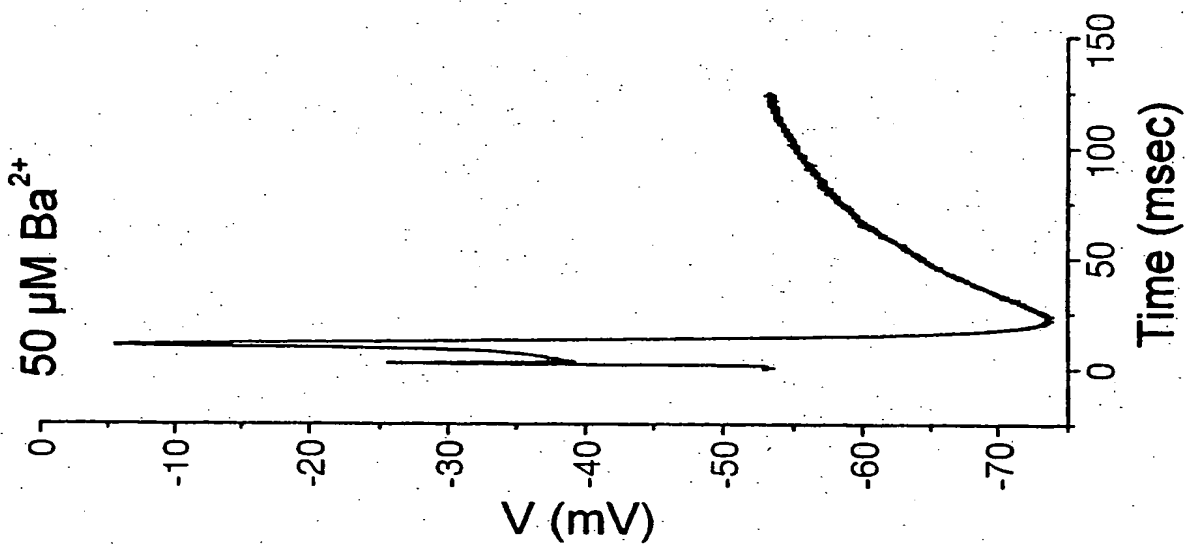
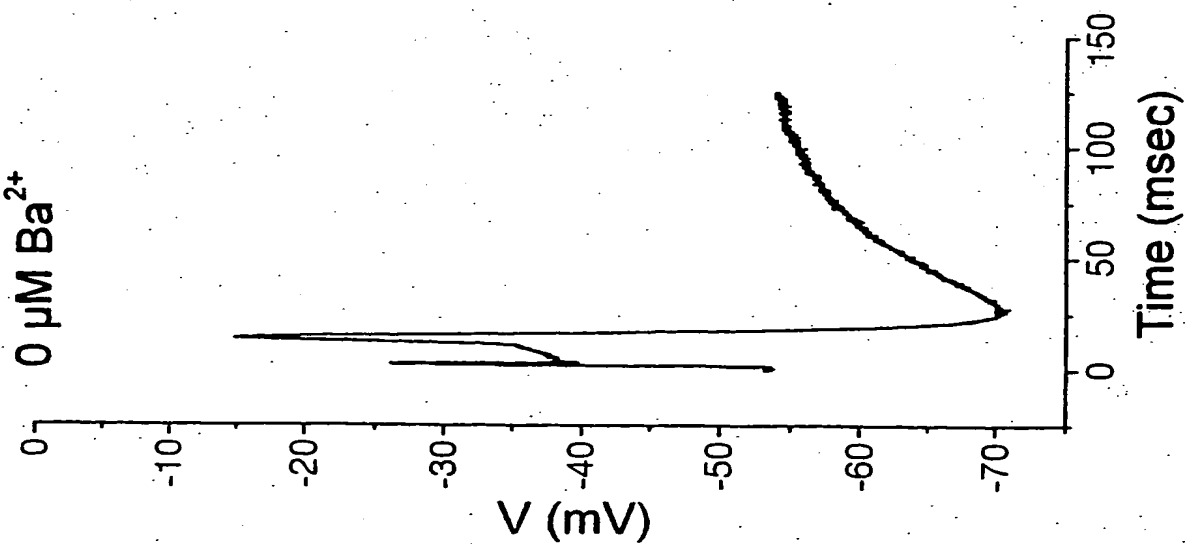


FIG. 3A

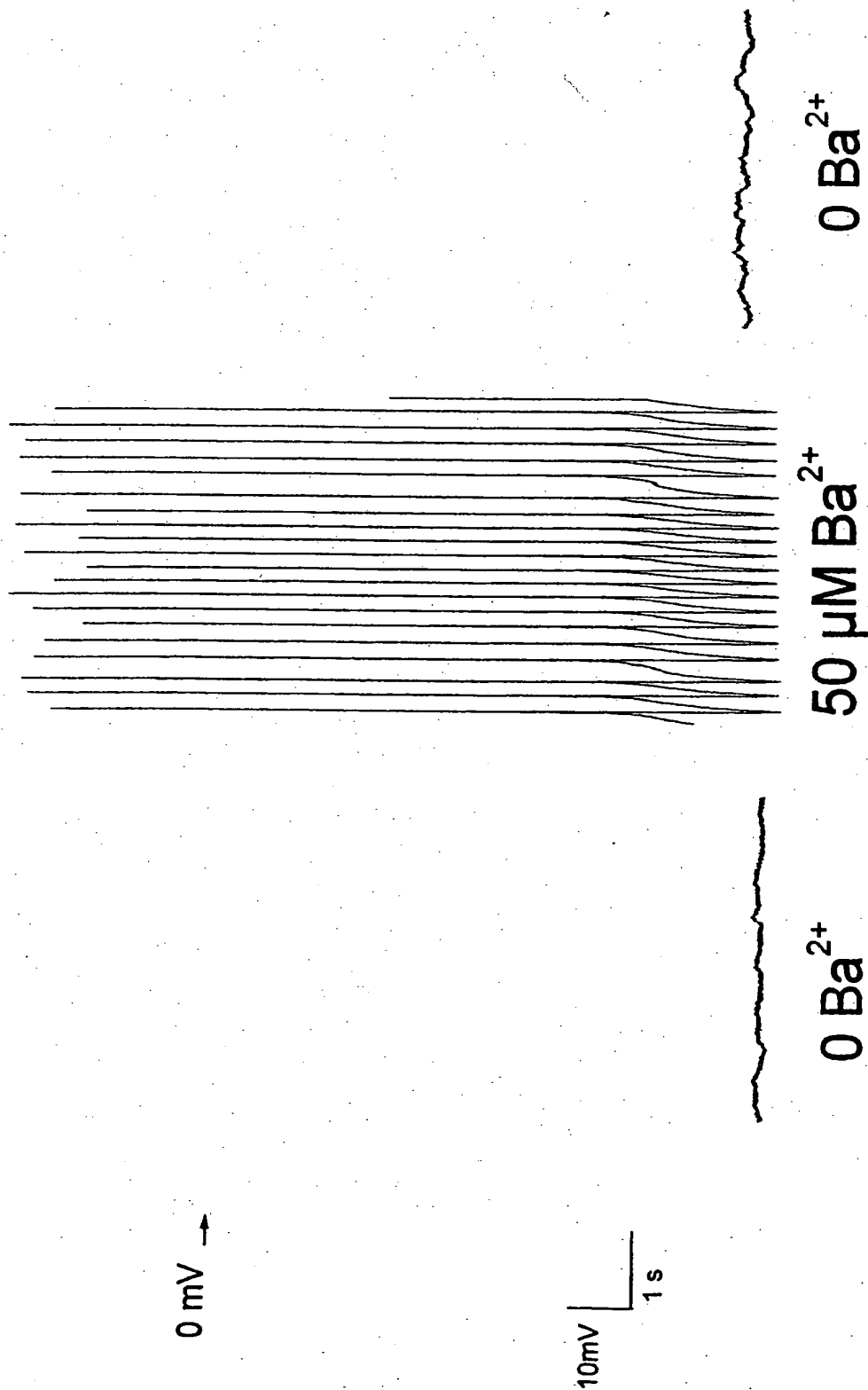


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FIG. 3I

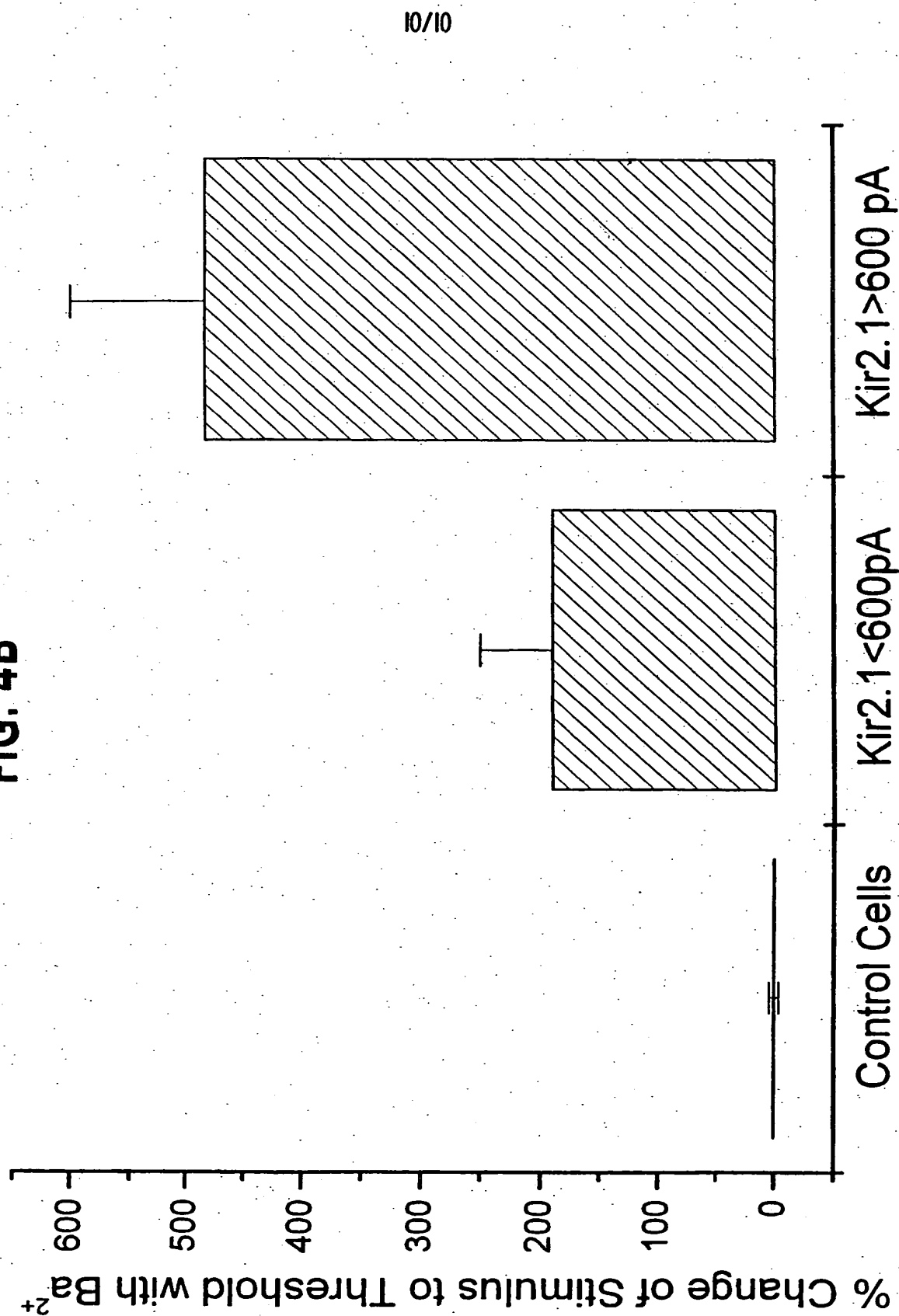
FIG. 3H

FIG. 3G



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FIG. 4B



## INTERNATIONAL SEARCH REPORT

Inter      nal Application No  
PCT/US 99/22468

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NO ET AL: "ECDYSONE-INDUCIBLE EXPRESSION IN MAMMALIAN CELLS AND TRANSGENIC MICE" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, USA, vol. 93, 1996, pages 3346-3351, XP002136440 page 3346 abstract page 3347; figure 2	35-37
A	NUSS H B ET AL: "Reversal of potassium channel deficiency in cells from failing hearts by adenoviral gene transfer: A prototype for gene therapy for disorders of cardiac excitability and contractility." GENE THERAPY 1996, vol. 3, no. 10, 1996, pages 900-912, XP000891388 ISSN: 0969-7128 cited in the application page 900 abstract	
A	HEBERT STEVEN C: "General principles of the structure of ion channels." AMERICAN JOURNAL OF MEDICINE JAN., 1998, vol. 104, no. 1, January 1998 (1998-01), pages 87-98, XP000891478 ISSN: 0002-9343	
A	JOHNS DAVID C ET AL: "Suppression of neuronal and cardiac transient outward currents by viral gene transfer of dominant-negative Kv4.2 constructs." JOURNAL OF BIOLOGICAL CHEMISTRY DEC. 12, 1997, vol. 272, no. 50, 12 December 1997 (1997-12-12), pages 31598-31603, XP002136441 ISSN: 0021-9258 cited in the application page 31598 abstract page 31602, paragraph 2 -page 31603, paragraph 1	

-/-

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 99/ 22468

### Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
**Remark: Although claims 1-35 partially insofar as such relate to in vivo methods, are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.**
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

### Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

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